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REVIEW

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"Bicellar" lipid mixtures as used in biochemical and biophysical studies

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Abstract Over the past decade "bicellar" lipid mixtures composed of the long-chain dimyristoyl phosphatidylcholine (DMPC) and the short-chain dihexanoyl PC (DHPC) molecules have emerged as a powerful medium for studying membrane associated, biologically relevant macromolecules and assemblies. Depending on temperature, lipid concentration and composition these lipid mixtures can assume a variety of morphologies, some of them alignable in the presence of a magnetic field. This article will examine the biophysical studies that have elucidated the various morphologies assumed by these lipid mixtures, and their use in the biochemical studies of biomolecules.

Introduction

Depending on the method by which a mesophase is formed, liquid crystals can be classified as being either thermotropic or lyotropic. Thermotropic mesophases are typically formed by molecules which are geometrically anisotropic and the phase formed is characteristic of the temperature. Note however, that the different scenarios of forming a liquid crystalline phase by either heating or cooling a substance are not equivalent, and both are not always equally possible.

Lyotropic liquid crystals are normally formed by molecules which contain chemical groups with differing properties and/or solvent affinity. Amphiphilic molecules, like phospholipids found in biological systems, have one part which prefers to associate with water and another which tends to be excluded from water. The driving force in this case is the hydrophobic effect (Tanford 1980). The phases formed depend strongly upon the concentration of the molecules, temperature, and the nature of the solvent.

The two classes of structures formed by both thermotropic and lyotropic liquid crystals are the so-called nematic (Fig. 1A and B) and smectic (Fig. 1C) phases . The nematic phase exhibits one-dimensional ordering, the lowest of all the liquid crystal classes. The direction of anisotropy, either in molecular shape or property, is fixed in space when all of the molecules adopt a similar orientation. However, the center of masses of the molecules are still able to diffuse freely in space. In a chiral nematic phase (Fig. 1B), commonly referred to as the cholesteric phase, the anisotropic vector rotates through space as a result of chiral centers within the constituent molecules.

Smectic phases (Fig. 1C) have two degrees of order; not only do the molecules adopt an average orientation, but they also arrange themselves into layers. Liquid-like motion is restricted to within the planes of these layers. This increased order means that this phase, compared to the nematic, exhibits "solid-like" behaviour.

Lyotropic nematic phases are formed by the long-range orientational ordering of the symmetric axis of non-spherical micelles, as shown in Fig. 1A. This alignable mesophase was first reported by Lawson and Flautt (1967) when they added appropriate alcohols to aqueous solutions of ionic surfactants, specifically a mixture of C_8 or C_{10} alkyl sulfate and its corresponding alcohol, sodium sulfate and water. Since this initial observation, the following decade saw numerous experimental and theoretical efforts to elucidate the morphologies and statistical mechanics governing lyotropic liquid crystalline behaviour (e.g., Amaral et al. 1979; Amaral et al. 1980; Forrest et al. 1980; Fujiwara and Reeves 1980; Charvolin 1984; Saupe 1984). [1]

Besides aqueous solutions of ionic surfactants and alcohols, many other ternary solutions form nematic structures

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[1] For a concise review of the early literature, the reader is referred-to the article by Forrest and Reeves (1981).

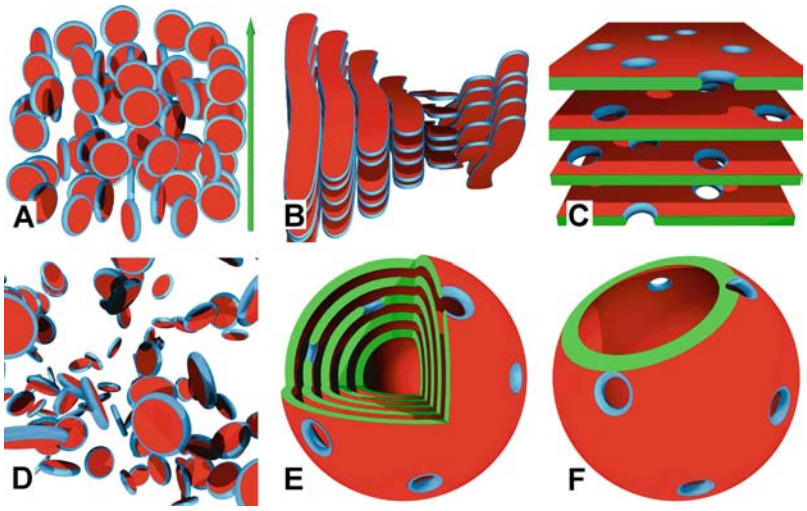

Fig. 1 Morphologies formed by dimyristoyl phosphatidylcholine (DMPC)/dihexanoyl PC (DHPC) lipid mixtures. **A** A nematic phase of bicelles oriented such that, on average the bilayer normals **N** are all pointing at right angles to the externally applied magnetic field (arrow), but the positions of the discs are random. In the literature, this orientation of bicelles was commonly referred to as "negatively aligned". **B** In a cholesteric or chiral nematic phase, the molecular assemblies twist slightly from one layer to the next resulting in a helical formation with a characteristic pitch. On the other hand, the spacings between bilayers lack a well-defined repeat distance, consistent with a nematic phase. The increased viscosity observed in this phase is the result of the entaglement of the elongated bilayered micelles. **C** Extended perforated lamellae, or smectic phase, exhibit both long range positional and orientational order, and can be equated to 1D quasi solids in one direction and 2D liquids (i.e., molecules freely diffusing within the bilayer) in the two orthogonal directions. The appearance of this phase is opaque and fluid. **D** An isotropic dispersion of bicelles. In this phase the bilayered micelles are randomly distributed in the water solvent, exhibiting no long range positional or orientational order. The solution is generally of low viscosity and colorless, and the transition from isotropic to nematic is first order. **E** Multilamellar and **F** unilamellar vesicles (ULV). For all morphologies shown, the colors red, blue and green correspond to the lipids DMPC, DHPC, and the bilayer's interior (hydrocarbon chains), respectively

over a relatively narrow range of concentration and temperature. These mixtures include sodium dodecyl sulphate (SDS) (Saupe 1984) and potassium laurate (KL)–decanol–water (Saupe 1984) mixtures. The most extensively studied of these being cesium per-fluoro-octanoate (CsPFO) (Boden et al. 1979; Boden and Holmes 1984; Boden et al. 1984).

In the case of thermotropic liquid crystal systems, a nematic-to-smectic transition is a common occurrence (Parmar and Clark 1989; Qiu and Ho 1990; Chen et al. 1991; Safinya et al. 1991; Lelidis and Durand 1994). However, the same cannot be said in the case of lyotropic systems. Observations of this transition have been few and include, as a function of temperature, those in CsPFO/water (Boden et al. 1979; Boden and Holmes 1984; Boden et al. 1984), ternary decyl ammonium chloride (DACL)–water–ammonium chloride mixtures (Holmes and Charvolin 1984), and tobacco mosaic virus (Dogic and Fraden 1997).

Liquid crystals in biological systems include the amphiphilic lipids (Fig. 2) of cellular membranes, proteins and molecules such as the DNA in chromosomes. These molecules can adopt a multiplicity of mesophases, some of which may be crucial for biological function. One such example is the plasma membrane which serves as the interface between the interior of the cell and the extracellular fluid that surrounds all cells.

Because they are one of the main components of biological membranes, amphiphilic phospholipids are of fundamental importance to biology. Phospholipids are composed of hydrophobic fatty acid chains and hydrophilic headgroups (Fig. 2), and along with cholesterol are the primary constituents of the cell membrane. In purified forms, lipid/water systems form a variety of interesting structures (e.g., lamellae, cubic and hexagonal phases, "rippled" bilayers, etc.) which for numerous reasons have been the focus of both experimental (Smith et al. 1988; Katsaras and

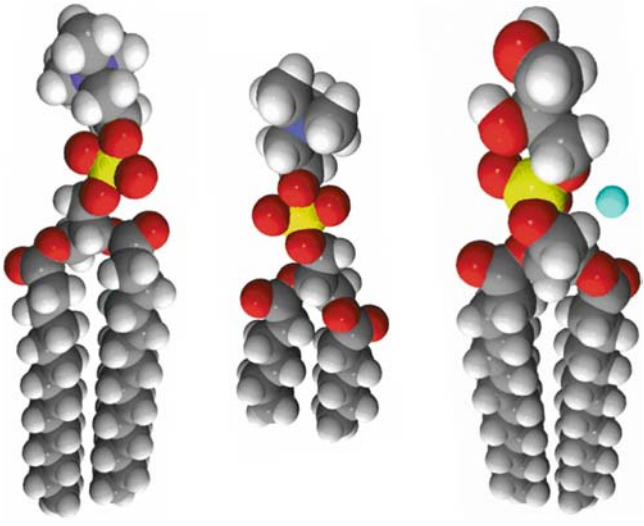

Fig. 2 Space filling models of lipids used in bicellar mixtures. The zwitterionic lipid DMPC (left) has two 14:0 hydrocarbon chains while the "detergent-like" DHPC (center) contains two 6:0 chains. The hydrocarbon chains are hydrophobic and sequester in a manner to exclude water. The negatively charged DMPG (right) differs from the neutral DMPC only in that it possesses a glycerol rather than a choline hydrophilic headgroup. The Na^+ counter ion accompanying DMPG is also shown

Stinson 1990; Raghunathan and Katsaras 1995; Katsaras and Raghunathan 1995; Katsaras 1998; Pozo-Navas et al. 2003; Harroun et al. 2004) and theoretical interest (Helfrich 1973; Helfrich 1978; Lipowsky and Leibler 1986; Mutz and Helfrich 1990; Lipowsky 1991; Lubensky and MacKintosh 1993; Nagle and Katsaras 1999).

One such structure, consisting of phospholipids and small amounts of detergent is the disk-shaped micelle, commonly referred to as the "bicelle" (Ram and Prestegard 1988; Sanders and Prestegard 1990; Sanders et al. 1993), previously observed in aqueous solutions of ionic surfactants and alcohols (Lawson and Flautt 1967; Amaral et al. 1979; Amaral et al. 1980; Forrest et al. 1980; Fujiwara and Reeves 1980; Charvolin 1984; Saupe 1984; Forrest and Reeves 1981). In this system, a typical saturated acyl chain lipid, such as dimyristoyl phosphatidylcholine (DMPC) shown in Fig. 2, forms a disk-shaped bilayer, whose edges are stabilized by a curved monolayer of detergent (Sanders and Prestegard 1990; Sanders et al. 1993). For biologists, however, a more pertinent system is the one whereby the detergent has been substituted by a short chain phospholipid, such as dihexanoyl phosphatidylcholine (DHPC), also shown in Fig. 2 (Sanders and Schwonek 1992; Sanders et al. 1994). Since their discovery, bicelles have been used in a number of studies attempting to elucidate the structure of proteins under physiologically relevant conditions (Sanders et al. 1994; Tjandra and Bax 1997; Sanders and Landis 1995; Struppe et al. 1998).

The canonical recipe of bicelle mixtures is the binary composition of DMPC and DHPC. Prior to recent structural studies, the magnetically alignable aggregate morphology was surmised, based on nuclear magnetic resonance (NMR) studies, to be the bicelle (Sanders and Schwonek 1992; Sanders et al. 1994; Tjandra and Bax 1997; Sanders and Landis 1995; Struppe et al. 1998; Prosser et al. 1996), and continues to this day as the model used to interpret much of the NMR spectroscopic data.

^{31}P and ^{2}H-NMR techniques have contributed extensively to much of the information presently known about bicellar mixtures. The quadrupolar splitting of ^{2}H (spin 1 nucleus) energy states is orientationally dependent, thus the orientation with respect to the applied magnetic field **B** of ^{2}H-labeled bonds can be determined. Therefore, ^{2}H labeling of the lipid acyl chains can be an effective measure of the alignment of the lipid bilayer in the magnetic field (Fig. 3A–C). The line shape of the 100% naturally abundant ^{31}P (spin 1/2 nucleus) signal indicates the relaxation time and thermal motion of the lipid molecules, and by inference the morphology. In addition, the appearance of more than one ^{31}P chemical shift may indicate the segregation of the different lipid in the mixture, as shown in Fig. 3D. For a recent review of bicellar mixture studies from a strictly NMR perspective, the reader is referred to Marcotte and Auger (2005).

Although NMR can be employed to deduce the molecular organization of molecules, it is a technique best suited to study the short range order and dynamics of model and biological membranes. Since short range ordering is unique to the various mesophases (i.e., lamellar, hexagonal, etc.),

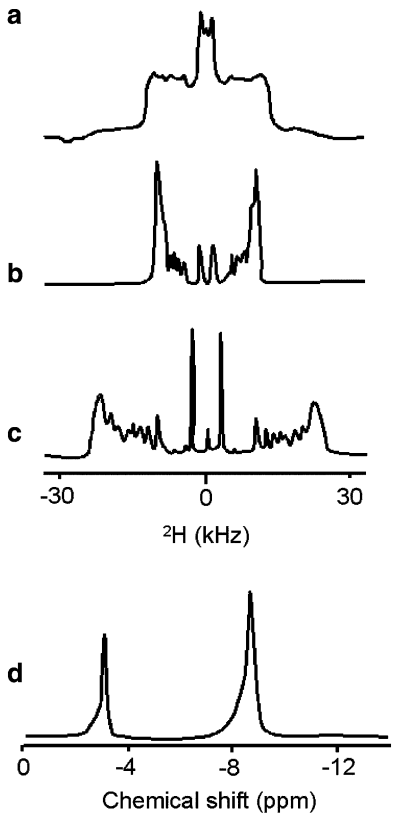

Fig. 3 ^{2}H and ^{31}P-NMR spectra adapted from (Howard and Opella 1996) and (Marcotte and Auger 2005), respectively. **a** ^{2}H-NMR powder pattern spectrum of randomly dispersed 50% wt/v chain perdeuterated $DMPC_{d54}$ MLV, **b** 3.4/1 [DMPC]/[DHPC] mixture (22% wt/v) and 0.25% molar uniformly ^{15}N-labelled fd coat protein, and **c** same as **b** but with the addition of 5% molar Tm^{3+}. ^{2}H-NMR spectra without (not shown) the fd coat protein are similar. For the nondoped aggregates **b**, their bilayer normals **n** are perpendicular to the applied magnetic field, while the Tm^{3+} doped aggregates **c** have their **n** parallel to the magnetic field. It is evident that orienting the lipid assemblies leads to a dramatic increase in spectral resolution, with deuterated sites along the DMPC's hydrocarbon chains giving rise to a doublet (**a** vs. **b**). Doping the aggregates with Tm^{3+} ions **c** leads to the observed quadrupole splitting increasing by a factor of two. **d** ^{31}P-NMR spectrum of bicellar mixture aggregates at 37°C with **n** oriented perpendicular to the magnetic field

^{31}P-NMR has been used to establish their presence. However, mesophases determined by NMR are a reflection of the relaxation times of the lipid molecules, and as such, different isotropic phases (e.g., Fig. 1D) cannot be identified. For example, distinguishing a "positively aligned" nematic from a smectic phase is problematic since in both cases their bilayer normals **N** are parallel to the applied magnetic field **B**.

On the other hand, scattering techniques such as small-angle neutron scattering (SANS) and traditional Bragg diffraction lend themselves ideally to determining the global assembly of membrane systems. A technique such as SANS is suitable for structural investigations on length scales between 10 and 3000 Å and typical samples consist of macromolecules or assemblies of molecules in solution. SANS provides information on both the size and shape of the objects in situ as well as their inter-particle

interactions. However, unlike traditional crystallography, there is no requirement for crystalline samples.

This article will describe studies that over the years have demonstrated the rich variety of morphologies exhibited by bicellar lipid mixtures, and which are still being vigorously studied. Interpretations of the results produced by different experimental techniques has led to apparent discrepancies between studies in which assignments of various morphologies to phases have been made. We show that much progress in the proper understanding of the standard bicellar mixture (i.e., DMPC/DHPC) has been made, and that this may point the way to the development of more complex and biologically pertinent mixtures. In the final section, we will outline some of the uses of bicellar mixtures in biochemical studies of biomolecules.

Structural characterization studies of bicellar lipid mixtures

In the following discussion, we shall refer to the typical DMPC/DHPC binary mixture as "bicellar", regardless of the morphology, and whose molar ratio we define to be $Q =$ [DMPC]/[DHPC]. We shall also discuss "doped" bicellar mixtures, where charged additives are either multivalent cations (e.g., Tm^{3+}, Eu^{3+}, Yb^{3+}, etc.) or negatively charged lipids such as, dimyristoyl phosphatidylglycerol (DMPG). In this case, the molar ratio of charged species to DMPC is given by R.

Studies of nondoped bicellar lipid mixtures

The interpretation of the first NMR studies of bicellar systems were that both lipid/detergent and pure lipid bicellar mixtures form a nematic phase induced by a magnetic field **B**, which preferentially aligns the morphology's bilayer normal **N** to be perpendicular to **B** (Fig. 3). In this case, the nematic order parameter is defined as,

$$P_2 = \langle (3\cos^2(\theta) - 1)/2 \rangle = -1/2,$$

where θ is the angle between the nematic order director and the bilayer normal. This isotropic to nematic transition is caused by negative anisotropy of the magnetic susceptibility, and is termed in the literature as "negative alignment" (Hare et al. 1995; Katsaras et al. 1997). Although these aggregates possess long-range orientational order they nevertheless lack positional order, which is typical of a nematic phase.

The first temperature–lipid composition phase diagram of nondoped bicellar mixtures was constructed by Raffard et al. (2000) (Fig. 4A). Using ^2H and ^{31}P solid state NMR they determined that for 20 wt% lipid mixtures and over a range of temperatures (T = 25–45°C), bicelles existed at Q ranging from 6.7 to 2.6. In the presence of 100 mM KCl, bicelles presumably formed over the same temperature range, but at a slightly different Q range (6.7–1.9). The lipid assemblies in 100 mM KCl also exhibited a higher degree

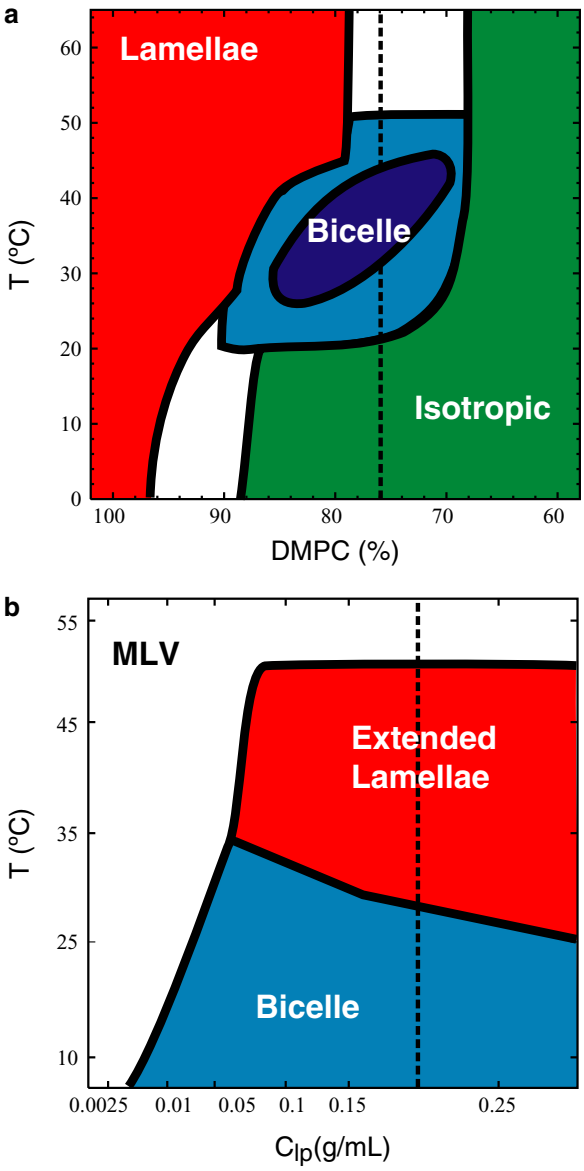

Fig. 4 Initial phase diagrams of nondoped bicellar mixtures. **a** Temperature-composition phase diagram of DMPC/DHPC mixtures in 80 wt% D_2O adapted from the ^{31}P-NMR studies of Raffard et al. (2000). **b** Temperature-lipid concentration phase diagram of $Q = 3.2$ in varying amounts of D_2O adapted from the SANS studies by Nieh et al. (2002). The phase diagrams are comparable only at a trajectory (dashed line) corresponding to DMPC ~76% for the Raffard phase diagram **a** and lipid concentration (c_{lp}) ~0.2 for the Nieh et al. phase diagram **b**

of macroscopic orientation. For $Q = 3.5$, the temperature range where bicelles were observed was greatest at 40 wt% water and nonexistent for ≥ 95 wt%. Besides bicelles, Raffard et al.'s study also identified regions of lamellar and isotropic phases (see Fig. 1) in both KCl and salt free systems. From an NMR perspective, an isotropic phase is the result of small objects undergoing fast isotropic motion. Since NMR cannot differentiate between micelles and fast tumbling bicelles, the precise morphology of aggregates within the isotropic phase remained unresolved. In general, extended lamellae were found to exist at higher

DMPC/DHPC mole fractions (e.g., $Q > 6.7$) and higher temperatures, while the isotropic phase was observed for $Q < 1.9$ mixtures and lower temperatures. In a combined ^{1}H-NMR and SANS study, Luchette et al. (2001) determined that for $0.2 < Q < 1.0$ and a range of temperatures (10–40°C), the observed isotropic, or so-called "fast tumbling phase", was consistent with the bicelle morphology.

Bolze et al. (2000) reported on small-angle x-ray scattering (SAXS) and ^{31}P-NMR studies of $Q = 2.6$ mixtures as a function of water content, between 20 and 40°C. Below 25°C there was no stacking of aggregates purported to be bicelles or ULV in the direction parallel to the bilayer normal, and no alignment due to an applied magnetic field. However, at high lipid concentrations (8–30% w/v) and temperatures above 25°C, magnetic alignment was observed and was associated with the formation of bilayer stacks. MLV are proposed to form at lipid concentrations <1% w/v above 25°C.

In another solid state ^{2}H and ^{31}P-NMR study, Sternin et al. (2001) reported on the temperature induced morphologies of $Q = 4.5$ and $c_{lp} = 22.7$ wt%. Below 21°C they observed two coexisting micelle species; one composed of the bicellar mixture and another of pure DHPC. Between 22 and 24°C the DMPC and DHPC molecules demixed with the DMPC molecules forming lamellae and the DHPC forming micelles. Increasing the temperature to between 26 and 32°C caused the DMPC hydrocarbon chains to "melt" and induce an exchange of lipid molecules between the micellar and lamellar phases, with DHPC molecules migrating to the DMPC lamellar phase, and vice versa. According to Sternin et al., bicelles form only over a narrow temperature range, between 32 and 36°C. Above 36°C there is a coexistence of micelles, bicelles and lamellae.

A temperature-lipid concentration phase diagram was constructed by Nieh et al. using SANS data obtained from $Q = 3.2$ mixtures dissolved in deuterium oxide (Fig. 4B) (Nieh et al. 2002), where the total lipid concentrations ranged from 0.0025 g/ml (~0.23 wt%) to 0.25 g/ml (~22.72 wt%) and temperatures between 10 and 55°C. Whereas Raffard et al. (2000) maintained a constant lipid concentration and varied Q, Nieh et al. kept Q constant and varied the concentration. Therefore, the phase diagrams are only comparable at a trajectory through the Raffard et al. phase diagram corresponding to $Q = 3.2$, or DMPC = 76% (dashed line through Fig. 4A), or c_{lp} ~0.2 in the Nieh et al. phase diagram (dashed line through Fig. 4B). At low temperature, the so-called isotropic phase, as deduced by NMR, was shown by SANS to be made up of an isotropic suspension of monodisperse bicelles. This corresponds to the isotropic phase observed by NMR, which, as discussed earlier, is not capable of differentiating between micelles and fast tumbling bicelles. According to Nieh et al., bicelles were found to persist until 25°C whereupon they transformed into magnetically alignable, extended perforated lamellae. The perforated lamellar morphology is made up of smectic bilayers, littered with pore defects induced by the short-chain DHPC molecules (Fig. 1C). DHPC molecules coat the edges of the pores to minimize the curvature energy. At approximately the same temperature, NMR data shows that the isotropic phase transforms into a magnetically alignable phase, which had been interpreted to be negatively aligned bicelles (Fig. 1A). Increasing the temperature further results in the formation of multilamellar vesicles (MLV), shown in Fig. 1E. Recently, a perforated lamellar morphology was observed by Soong and Macdonald (2005) using different types of NMR (i.e., ^{1}H, ^{2}H and ^{31}P-NMR) and $Q = 4.5$ mixtures doped with polyethylene glycol (PEG) lipids (e.g., dimyristoyl phosphatidylethanolamine-PEG 2000).

These apparently conflicting results were resolved by two recent studies. Using a combination of polarized light microscopy (POM) and SANS (Nieh et al. 2004a; Harroun et al. 2005), it was concluded that the phase space previously thought to be composed of bicelles (Raffard et al. 2000) or extended lamellar sheets (Nieh et al. 2002), is in fact a chiral nematic phase made up of worm-like, or ribbon-like, micelles (see Fig. 1B). This result was verified by van Dam et al. (2004) using cryo-transmission electron microscopy (cryo-TEM) in which they observed quasi-cylindrical, elongated micelles. This morphology of worm-like micelles is consistent with the frequent observations of increased viscosity in the temperature range where magnetic alignment is known to take place (Sanders and Schwonek 1992), and is the result of these elongated structures becoming entangled.

We speculate that the cross section of worm-like micelles is most probably that of a DMPC bilayer with edges coated by a DHPC monolayer. In this sense, the cross section is similar in profile to a bicelle. This may help explain why ^{31}P-NMR data, which shows the segregation of the DMPC and DHPC lipids, has been interpreted in terms of a bicelle model. However, cryo-TEM data seem to indicate the micelles to be nearly cylindrical. The diameter of the micelles seen by cryo-TEM and SANS are well below the ~100 Å radius of the typical bicelle for $Q > 2.0$ mixtures, strongly indicating that micelles are unlikely to form a columnar phase of stacked bicelles.

Summary

The recent report of Harroun et al. (2005) provides a nearly comprehensive study of bicellar mixture mesophases, reconciling a number of conflicting views. The phase diagram shown in Fig. 5 provides a new paradigm for the description and study of such phases. It is now clear that bicellar mixtures exhibit three dominant mesophases, and the application of techniques complementary to NMR was essential in identifying and characterizing these phases. It is important to note that, while the phases characterized using these techniques are different than those predicted by NMR, the phase boundaries identified are equivalent.

In contrast to previous interpretations of NMR data, it is now clear that for $Q = 3.2$ and 5.0 mixtures, bicelles populate the temperature region below ~20°C and over a wide range of c_{lp}. In the case of $Q = 2.0$, bicelles can be found at lower c_{lp} up to 60°C. The chiral nematic phase of worm-like micelles also features prominently in the Q= 3.2 and 2.0

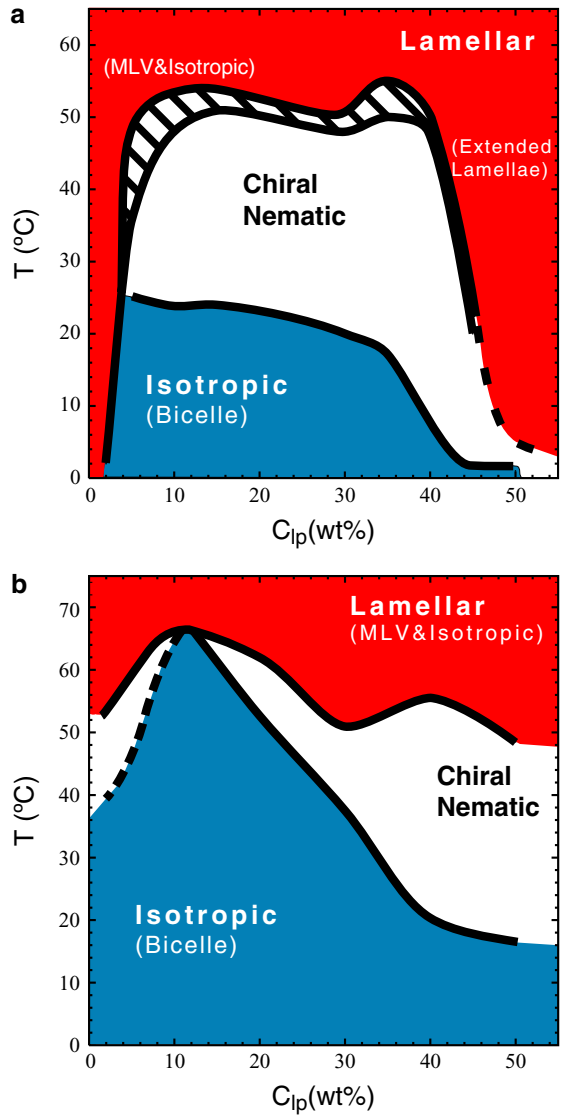

Fig. 5 Phase diagrams adapted from Harroun et al. (2005) of nondoped $Q = 3.2$ **a** and 2.0 **b** mixtures. The morphologies were determined using a combination of POM and SANS measurements. The dashed lines indicate ambiguity in determining the exact phase transition while the hatched area for $Q = 3.2$ **a** depicts the coexistence region of nematic and lamellar phases. The $Q = 3.2$ phase diagram **a** differs from the one by Nieh et al. (2002) (Fig. 4B) only to the extent that the extended lamellar morphology was shown to be a chiral nematic phase made up of worm-like micelles. The range of c_{lp} was also extended

phase diagrams. Finally, nondoped bicellar mixtures form MLV and/or extended perforated lamellae at temperatures $> 20°C$ and $c_{lp} > \sim 0.05$ g/ml.

Structural studies of Tm^{3+} and dimyristoyl phosphatidylglycerol (DMPG) doped bicellar lipid mixtures

Negatively aligned liquid crystalline aggregates (Fig. 1A) are not ideal for the study of larger, nearly immobile, membrane proteins, at least for the purposes of NMR investigations. Unless the protein in question undergoes fast axially symmetric motion around the bilayer normal, the resultant cylindrical NMR powder pattern is difficult to interpret. Thus, from a solid state NMR point of view, the practical size limit of proteins possessing multiple labels that can be studied in these nematic aggregates, is probably on the order of a few thousand daltons. A solution to this problem is the production of so-called "positively aligned" lipid aggregates, whereby $\langle \mathbf{N} \rangle$ is parallel to $\mathbf{B}$ (e.g., Figs. 1C and 3C).

Positively aligned aggregates were first produced by Prosser et al. (1996) by doping DMPC/DHPC mixtures with appropriate lanthanide ions, usually Tm^{3+}, Eu^{3+} or Yb^{3+}. The lanthanide ions bind to the lipid's hydrophilic headgroup, thereby imparting a significant positive magnetic susceptibility anisotropy to the lipid bilayer, such that $\langle \mathbf{N} \rangle$ assumes a parallel orientation with respect to $\mathbf{B}$ (3C). The interpretation of this result is that the bicelle nematic director flips from being perpendicular to $\mathbf{B}$, to being parallel, as shown in Fig. 1B, and $P_2 = 1$. Assuming that the membrane protein can be reconstituted with these positively aligned aggregates, in theory, there is no restriction to the size of a protein that can be studied with solid state NMR.

It was subsequently found, by neutron scattering experiments, that doping bicellar lipid mixtures with Tm^{3+} causes the system to undergo a transition from a negatively aligned nematic phase of bicelles to a smectic phase of extended lamellae (Fig. 1C). The liquid crystalline smectic bilayers were highly aligned with respect to $\mathbf{B}$ (mosaic spread of $\pm$ 1.0° FWHM) (Katsaras et al. 1997). The original NMR study on this system was then revisited by Prosser, and the observation that the positively aligned phase was smectic, rather than nematic bicelles, was confirmed (Prosser et al. 1998a).

A concern, however, of using lanthanides to create positively aligned, smectic aggregates is the possibility of the ions binding to the protein of interest, altering its conformation and most likely, its function. Moreover, in the case of NMR spectroscopy, the lanthanide ions are known to shift and broaden the NMR signal further complicating the interpretation of chemical shift resonances. A solution to this problem was arrived at by Prosser et al. (1998b) whereby bicellar mixtures were doped with a phospholipid chelate molecule (e.g., dimyristoyl phosphatidyl ethanolamine-diethyl enetriamine penta-acetate [DMPE-DTPA]) that preferentially binds lanthanide ions, thus practically eliminating the possibility of the other phospholipids or proteins directly coordinating the lanthanide ions. Prosser et al. (1999) have also shown by ^{2}H-NMR that the conformational change induced by Tm^{3+} ions to the surface associated leucin enkephalin protein is reversed by the addition of 1,11-bis[distearylamino]-DTPA. It should be noted, however, that the use of a negatively charged lipid, such as DMPG in combination with the chelate is either essential, or highly recommended in stabilizing the bicellar aggregates (Prosser et al. 1998b; Prosser et al. 1999), further adding to the system's complexity.

A first attempt in elucidating the morphological behaviour of a Tm^{3+} doped bicellar system ($Q = 3.2$, $R = 0.014$) was undertaken by Nieh et al. (2001). A number of lipid concentrations were examined at 10 and 45°C. Like the nondoped mixtures discussed in Section 2.1, at 10°C the Tm^{3+}-doped mixtures formed bicelles throughout the range of lipid concentrations examined. At 45°C and $c_{lp} >$ 4.5 wt% bicelles transformed into extended lamellae populated with perforations, a morphology once proposed by Prosser et al. (1998a).

At $c_{lp} < 4.5$ wt% monodisperse bicelles transformed into low polydispersity unilamellar vesicles (ULV) (Fig. 1F). The formation of low polydispersity ULV from monodisperse bicelles can be understood as follows; above a critical size the high edge energy of bicelles makes them unstable forcing them to "close" unto themselves, forming ULV. This is possible as long as the rate of closure is faster than the fusion of bicelles forming lamellae (Leng et al. 2003). Ca^{2+} doping produces very similar morphologies (Nieh et al. 2004b), although calcium ions will not have the same influence on the magnetic susceptibility as lanthanide ions.

In later studies DMPG was substituted for Tm^{3+} (Nieh et al. 2002; Nieh et al. 2004b). In the first of these ($Q = 3.2$, $R = 0.014$) the morphologies observed (Fig. 6A) were similar to those produced by doping with Tm^{3+}. However, no ULV were formed upon heating reasonably dilute DMPG doped bicellar mixtures. On the other hand, it is well known that increased charge density dampens bilayer fluctuations resulting in rigid lamellae (Higgs and Joanny 1990). This may account for why MLV, which have higher curvatures than extended lamellae, form in nondoped neutral bicellar mixtures (Nieh et al. 2002) and ULV, which have less curvature than MLV, form in weakly charged Tm^{3+} systems. In the case of doping with the negatively charged lipid DMPG, the bicelles most likely become so rigid that they can not fold unto themselves to form ULV. ULV were subsequently produced by either reducing the doped system's net charge by the addition of Tm^{3+} (Nieh et al. 2002), or simply by reducing the amount of DMPG ($R \leq 0.006$) (Nieh et al. 2004b).

A recent dynamic light scattering and SANS study by Yue et al. (2005) has confirmed that DMPG-doped bicellar mixtures spontaneously form low polydispersity ULV that are highly stable over a wide range of temperature and whose size is independent of c_{lp}. It was concluded that these highly stable ULV possess characteristics that may make them useful encapsulation devices suitable for pharmaceutical and biomedical applications. Rare is also the occurrence of spontaneously forming, stable ULV in phospholipid systems. Moreover, it is possible that these ULV represent the first lipid ULV in thermodynamic equilibrium (Leng et al. 2003).

Summary

In contrast to nondoped bicellar mixtures, a detailed study equivalent to the one by Harroun et al. (2005) has not yet been undertaken for doped bicellar mixtures. Presently, the most complete study of doped DMPC/DHPC mixtures is the one by Nieh et al. (2002), and whose adapted phase diagram is shown in Fig. 6. The published reports have identified the important role of surface charge density in modulating the observed liquid crystalline morphologies. In particular, a bicelle-to-ULV transition, which is not present in neutral bicellar mixtures, depends on imparting a small positive or negative charge density to the system.

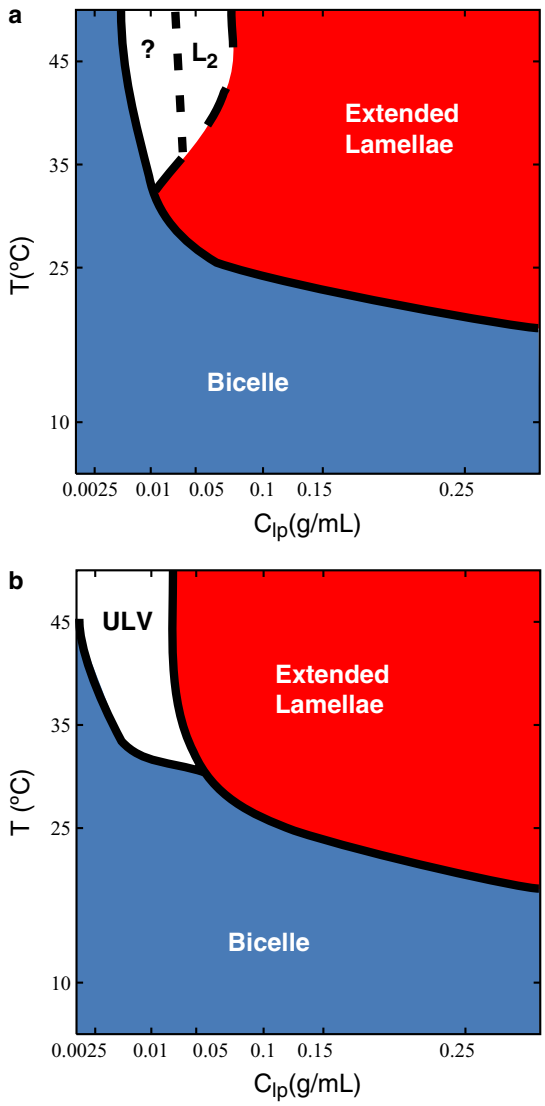

Fig. 6 Phase diagrams adapted from Nieh et al. (2002) of **a** DMPG-doped DMPC/DHPC ([DMPC]/[DHPC]/[DMPG] = 3.2/1.0/0.21) and of **b** DMPG and Tm^{3+} doped DMPC/DHPC ([DMPC]/[DHPC]/[DMPG] = 3.2/1.0/0.21). The morphologies shown were determined SANS

Applications of bicellar lipid mixtures

Use of bicellar mixtures to study membrane associated macromolecules

For NMR studies isotropic bicelles provide an ideal model system for high resolution investigations of membrane

peptides and proteins. The preference for the use of isotropic bicelles over micelles in NMR studies of membrane associated peptides is that the bicelle interior consists of a true lipid bilayer, something possibly necessary for the proper folding and activity of an integral protein and not achievable through the use of detergent micelles.

In the case of NMR experiments, the spectroscopic properties associated with samples whose $\langle \mathbf{N} \rangle$ is parallel to **B** are such that the spectra for each labeled site consist of single line resonances rather than "powder patterns". The observed splittings can then be related to the orientations of the individual bonds or atoms relative to **B**. Such samples thus provide a distinct advantage in structure determination.

One of the first studies undertaken to assess the feasibility and practicality of using bicelles to study membrane associated polypeptides was by Sanders and Landis (1995). Using bicellar mixtures of DMPC, and either DHPC or the bile salt 3-(chloramidopropyl) dimethyl ammonio-2-hydroxyl-1-propane sulfonate, commonly referred to as CHAPSO, they carried out ^{1}H and ^{13}C-NMR studies of 15 membrane associating peptides and proteins (e.g., gramicidin D, myelin basic protein, cytochrome *c*, melittin, alamethicin, β-amyloid, etc.). Sanders and Landis (1995) determined the macromolecule's ability to interfere with the magnetic orientation of the bicellar mixture aggregate, while the suitability of these aggregates as membrane mimics was determined by using the integral membrane enzyme, diacylglycerol kinase (DAGK). Of note were the high resolution ^{13}C-NMR spectra obtained from the cytochrome *c* and leucine enkephalin aligned assemblies. From the results of the various reconstituted systems, the authors concluded that bicellar mixture aggregates may be uniquely and effectively employed as membrane mimics to facilitate NMR structural studies of many membrane associated proteins.

Vold et al. (1997) demonstrated that for [DMPC]/[DHPC] between 0.5 and 1.0, complete high resolution ^{1}H-NMR spectra were obtained for small membrane associated peptides, such as mastoparan. Tjandra and Bax (1997) using nondoped bicellar mixtures at 25 and 38°C, corresponding to the isotropic (bicelles) and magnetically alignable phases (most likely worm-like micelles), respectively, were able to directly determine the distance and bond angles from dipolar couplings of human ubiquitin, a water soluble protein. These results were found to compare favorably with those predicted by the protein's crystal structure.

Another study of a reconstituted membrane associated protein into a magnetically alignable bicellar morphology was by Howard and Opella (1996). Using uniformly ^{15}N-labeled fd coat protein they examined the reconstituted protein in both negatively ($\mathbf{N} \perp \mathbf{B}$) and positively aligned ($\mathbf{N} \parallel \mathbf{B}$) lipid bilayers at T = 43°C. The study demonstrated that an integral protein can be magnetically aligned in both lanthanide doped and nondoped DMPC/DHPC bilayers. However, compared to nondoped bilayers, the uniaxially aligned lanthanide doped system gave rise to improved chemical-shift dispersions.

Using similar bicellar mixtures as Tjandra and Bax (1997), Losonczi and Prestegard (1998) improved the stability and orientation of the magnetically alignable assemblies by doping dilute bicellar solutions with small amounts of charged amphiphiles i.e., the positively charged hexadecyl(cetyl)trimethylammonium bromide (CTAB) and the negatively charged SDS. Besides improving stability and alignment, doping with these positively and negatively charged lipids also extended the temperature range in which they could be aligned.

Bicellar mixtures have also been developed to remain stable in pH conditions ranging from acidic to basic (Ottiger and Bax 1999) by replacing the carboxy-ester bonds present in DMPC and DHPC lipids with ether linkages, thus preventing the degradation of these compounds by acid- or based-catalyzed hydrolysis. It was shown that ubiquitin's dipolar couplings remained unaltered from pH = 2.3 to 10.4, demonstrating the robustness of both the protein and the bicellar's mixture morphology.

Cavagnero et al. (1999) developed an alignable lipid mixture which differed from the one by Ottiger and Bax (1999), extending the low pH range. The system was composed of 1,2-di-O-dodecyl-sn-glycero-3-phosphocholine (DIODPC) and the bile salt CHAPSO. For a [DIODPC]/[CHAPSO] = 4.3 mixture, the magnetically alignable assemblies were oriented over a range of pH, i.e. 1.0 to 6.5, and were chemically stable throughout. In future, this system may prove particularly useful for measuring residual dipolar couplings of macromolecules whose native state occurs in extreme acidic conditions.

Struppe et al. (2000) developed acidic bicellar mixtures by the addition of either dimyristoyl phosphatidylserine (DMPS) or DMPG (see Sect. 2), which were stable from pH 5.5 to 7.0. Compared to neutral bicellar mixtures, the addition of acidic (negatively charged) phospholipids makes the resultant system more biologically relevant and it can easily be prepared under a variety of conditions, including temperature, salt concentration and pH. By having neutral and acidic bicellar mixtures peptide-membrane interactions can be contrasted. This was done in the case of the N-terminal myristoylated 14 residue segment of pp60^{v-src}, which has a net charge of +4. Struppe et al. found that the degree of peptide myristoyl chain ordering in acidic mixtures is higher than the myristoyl chain of a neutral peptide derived from protein kinase A, most likely the result of stabilizing electrostatic interactions. A similar bicellar mixture was used by Marcotte et al. (2003) to study the interactions of methionine-enkephalin with different lipid headgroups.

In 2000, Czerski and Sanders (2000) showed the functionality of the integral membrane protein, DAGK in membranes formed using variable long-chain phospholipids (e.g., dilauroyl PC, DMPC and dipalmitoyl PC) and two different "detergent" components; DHPC or CHAPSO. In the case of DAGK catalytic activity, the bicellar mixtures containing the CHAPSO proved more effective than those mixtures with DHPC. Moreover, DAGK exhibited a preference for DMPC and dipalmitoyl PC (DPPC) bicellar mixtures compared to those containing the shorter chain lipid dilauroyl PC. Interestingly, DAGK catalytic activity was near optimal in mixed micelle or vesicle systems having decyl maltoside and bovine heart cardiolipin as the detergent and lipid components, respectively (Czerski and Sanders 2000).

In another structure-function study, Whiles et al. (2002) employed ^{31}P-NMR to examine the kinetic behaviour of cobra venom phospholipase A_2 toward a variety of bicellar mixture substrates. Compared to standard micellar substrates, the enzyme hydrolyzed the lipids of the various bicellar mixtures (e.g., DMPC/DHPC, DMPC/CHAPSO, didecanoyl phosphatidylcholine/DHPC) at comparable rates. Furthermore, the enzyme did not show a marked preference for the short- or long-chain phospholipids in the various bicellar mixtures.

Protein crystallization using bicellar mixtures

Membrane associated proteins are known to constitute approximately one third of all known proteins (Goffeau 1995). Despite this, fewer than 100 membrane protein structures have been deposited with the RCSB (Research Collaboratory for Structural Bioinformatics) protein data bank. The primary reason for this scarcity of membrane protein structures is that integral proteins are not easily crystallized by standard techniques, and tend to aggregate in the course of crystallization from solution. However, when co-dissolved with lipids and/or detergents, membrane proteins co-crystallize with the detergent in their native structure (Caffrey 2003).

The first applications of this technique led to the crystallization of porin (Garavito and Rosenbusch 1980) and bacteriorhodopsin (Michel and Oesterhelt 1980). However, the first membrane protein structure determined to near atomic resolution was that of the photosynthetic reaction center of *Rhodopseudomonas viridis*, a purple sulphur photosynthetic bacterium whose 3D structure was solved by Deisenhofer et al. (1985). For their epic work, Deisenhofer, Huber and Michel were awarded the 1988 Nobel prize in chemistry.

In the case of the photoreaction center of *Rhodopseudomonas viridis* (Deisenhofer et al. 1985) and other membrane associated proteins (Garavito and Rosenbusch 1986; Cowan et al. 1995; Dutzler et al. 1999), the role of detergents in creating high quality crystals was essential. However, this approach is not applicable to all membrane associated proteins (Chiu et al. 2000) as many of them are unstable outside of a lipid membrane (Bowie 2001). A more appropriate environment to crystallize proteins is thought to be a lipid matrix, where the proteins are more likely to retain their native properties. For example, the addition of the lipid dioleoyl PC to a detergent solubilized protein, namely the cytochrome b_6 *f* complex of oxygenic photosynthesis, resulted in a dramatic improvement in crystallization efficiency (Zhang et al. 2003).

Landau and Rosenbusch (1996) crystallized bacteriorhodopsin (bR) using a bicontinuous lipid cubic phase. Based on their initial work, a bR x-ray structure was later obtained by them and others using microcrystals grown in a monoolein-based cubic phase (Pebay-Peyroula et al. 1997; Luecke et al. 1998; Luecke et al. 1999; Belrhali et al. 1999). Employing a bicontinuous lipid phase matrix, the protein is encased in a natural bilayer environment, yet is allowed to diffuse throughout the sample creating crystal nuclei. Functionally, these microcrystals were also shown to be indistinguishable from bR in native purple membrane, since upon photoexcitation they underwent the identical photocycle (Heberle et al. 1998). This methodology of cubic phase crystallization has also proven to be effective in crystallizing small molecules and water soluble proteins (Landau et al. 1997; Rummel et al. 1998). Nevertheless, it is a method not without its problems (Faham and Bowie 2002).

As shown in a previous section, bicellar mixtures can assume a variety of morphologies making it possible for proteins to access a number of lipid bilayer structures simply by varying temperature or lipid composition (see Sect. 2). Recently, Faham and Bowie (2002) have taken advantage of the morphologies offered by bicellar lipid mixtures and crystallized bR, solving the 3D structure by molecular replacement, and refining the data set to a resolution of 2.0 Å. Interestingly, they found that individual bR subunits are arranged in an anti-parallel fashion whereby, each monomer is surrounded by three other monomers. This result is in contrast to the cubic lipid phase bR crystals where the protein was found to be organized as parallel trimers. Although the advantages, in contrast to lipid cubic phase crystallization, offered by bicelle crystallization are not drastic, this study by Faham and Bowie (2002) does demonstrate that other methods of protein crystallization do exist and may eventually prove to be of use in crystallizing a broader class of membrane associated proteins.

Micellar electrokinetic chromatography

Recently, methods have been devised to develop low volume separation techniques (e.g., capillary column liquid chromatography and capillary electrophoresis) for high throughput screening (Holland and Leigh 2003) and to elucidate physiological processes (Mills and Holland 2004). Some of the advantages of such techniques are; small sample and mobile phase volumes, higher separation efficiency, as well as, lower cost devices and portability.

Micellar electrokinetic chromatography (MEKC) is a technique favorable to bioanalytical and pharmaceutical applications. Örnskov et al. (2002) reported on the immobilization of liposomes inside fused silica capillaries which were subsequently used as a nano-separation tool to study the interactions between small drugs and liposomes composed of DPPC and PS. This rapid and improved method is used to estimate lipophilicity, one of the parameters governing the success of potential drug candidates, and the screening of membrane associated peptides. Holland and Leigh (2003) and Mills and Holland (2004) extended this methodology by using DMPC/DHPC mixtures.

Concluding remarks

Over the last decade, DMPC/DHPC lipid mixtures have been extensively investigated using a number of techniques.

Based primarily on NMR data, the magnetically alignable phase for both doped and nondoped bicellar mixtures was surmised to be bilayered micelles, commonly referred to as bicelles. It was not until 2001 that SANS studies showed that magnetically alignable, Tm^{3+} doped bicellar mixtures did not adopt the much accepted bicelle morphology, but formed extended perforated lamellae (Nieh et al. 2001). The same group carried out another SANS study whereby, the negatively charged lipid DMPG, in the presence and absence of Tm^{3+} ions, was used to dope DMPC/DHPC mixtures (Nieh et al. 2002). The magnetically alignable morphology was also determined to be extended perforated lamellae.

The magnetically alignable morphology in nondoped systems has turned out to be more controversial. From numerous NMR studies, it was initially believed to be bicelles, but was later shown, by SANS, to be consistent with perforated lamellae (Nieh et al. 2002). However, most recently SANS, POM and cryo-TEM studies (Nieh et al. 2002; Harroun et al. 2005; van Dam et al. 2004) have shown that the magnetically alignable phase of nondoped bicellar mixtures is in fact characterized by worm-like micelles (Nieh et al. 2004a). The nondoped mixtures and their various morphologies have been used extensively in structure-function studies of membrane associated proteins and protein crystallization (see Sect. 3).

Clearly the phase behaviour of bicellar mixtures is much richer than previously assumed. It is only through the application of several complementary techniques (e.g. POM, SANS, NMR and cryo-TEM) that a nearly complete picture of bicellar phase behaviour could be developed. The composition, net charge and lipid concentration all play significant roles in determining the variety of phases and morphologies observed. As applications of these mixtures expand, and further compositions are required and explored, there is a clear need for continued investigations and characterizations of the phase behaviour and morphologies of these enigmatic lipid mixtures.

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